JACC Vol. 27, No. 3
March 1, 1996:560–6

Platelet-Dependent Thrombin Generation in Patients With Diabetes Mellitus: Effects of Glycemic Control on Coagulability in Diabetes

ISAO AOKI, MD, KATSUYA SHIMOYAMA, MD, NOBUO AOKI, MD, MASASHI HOMORI, MD, ATSUO YANAGISAWA, MD, KAZUHIKO NAKAHARA, MD, YOHKO KAWAI, MD,* SHIN-ICHI KITAMURA, MD,† KYOZO ISHIKAWA, MD, FACC

Tokyo, Japan

Objectives. **This study sought to assess the usefulness of platelet-dependent thrombin generation as an index of coagulability in diabetes and to determine the effect of glycemic control on coagulability in diabetes.**

Background. **It is important to investigate the interaction of platelets and the coagulation factors to clarify the processes of the coagulation system in detail.**

Methods. **Platelet-rich plasma (150 × 10^9/liter), 0.5 ml, was prepared, and 40 mmol/liter of calcium chloride was added to initiate clotting. S-2238 was added to each sample in a microtiter plate every 10 min, and the absorbance of the released color product at 2 min was measured spectrophotometrically at a wavelength of 405 nm using a microtiter plate reader as thrombin generation. We measured the platelet-dependent thrombin generation in patients with non–insulin-dependent diabetes mellitus grouped according to glycemic control.**

Results. **Platelet-dependent thrombin generation at 30 min after calcium chloride addition was significantly higher in 23 patients with poorly glycemic-controlled non–insulin-dependent diabetes mellitus without complications, such as diabetic retinopathy, nephropathy and neuropathy (hemoglobin [Hb] A_{1c} ≥9.0%) than in 46 healthy normal subjects (448 ± 75 vs. 165 ± 28 mU/min, $p < 0.001$). Thrombin generation in 31 well controlled diabetic patients without complications (Hb A_{1c} <9.0%) was intermediate (240 ± 72 mU/min) between those of the poorly controlled group and healthy normal subjects. Platelet-poor plasma from diabetic patients increased platelet-dependent thrombin generation in normal subjects.**

Conclusions. **Coagulability is evidently enhanced in patients with non–insulin-dependent diabetes mellitus compared with that in healthy normal subjects on the basis of assessments of the platelet-dependent thrombin generation, and good glycemic control may help to correct a hypercoagulable state in diabetic patients.**

(J Am Coll Cardiol 1996;27:560–6)

Diabetic angiopathy is classified into microangiopathy, which is specific to diabetes mellitus, and macroangiopathy, which is not specific to diabetes mellitus but is more common in diabetic patients than in the nondiabetic population. Because systemic atherosclerosis underlies macroangiopathy, macroangiopathy can cause myocardial infarction or cerebral infarction and thus have a significant impact on quality of life and survival. Recently, the blood coagulation system has been considered to be involved in the etiology of macroangiopathy (1). Many investigators have presented data showing that diabetic patients have enhanced platelet function (2–9) and hypercoagulability (10–18). However, it has also been reported that the plasma level of fibrinopeptide A, a direct indicator of coagulation, is normal in diabetic patients (19,20) and that the plasma level of antithrombin-III is normal (21,22) or increased (3). Thus, no definitive trend has been documented with respect to coagulability in diabetes. This lack is thought to be because coagulability was assessed using plasma samples in all these studies and because coagulation markers such as antithrombin-III and thrombin–antithrombin-III complex are only indirect indicators of thrombin generation.

It is known that thrombin generation occurs on the activated platelet surface very efficiently, and in turn, small amounts of thrombin serve to activate platelets (23). Therefore, investigation of thrombin generation on the platelet surface appears to be important for understanding the process of coagulation, and such measurements may be more useful for the investigation of coagulation cascade than the assessment of plasma fibrinopeptide A or TAT levels. Aronson et al. (24) focused on this point and developed an experimental system for the direct measurement of thrombin generation on platelets. Measurement of thrombin generation using the assay of Aronson et al. appears to be useful for investigating the local state of coagulation in the vessels. In the present study, we therefore measured thrombin generation by this method in diabetic patients classified by the control status of blood glucose and assessed the usefulness of platelet-dependent thrombin generation as an index of coagulability in diabetes.

From the Second Department of Internal Medicine and Department of Clinical Pathology, Kyorin University School of Medicine; *Central Clinical Laboratories, Keio University School of Medicine; and †Department of Internal Medicine, Mukojima Saiseikai Hospital, Tokyo, Japan.

Manuscript received March 15, 1995; revised manuscript received September 26, 1995, accepted October 17, 1995.

Address for correspondence: Dr. Kyozo Ishikawa, Second Department of Internal Medicine, Kyorin University School of Medicine, 6-20-2, Shinkawa, Mitaka-city, Tokyo 181, Japan.

0735-1097/96/$15.00
0735-1097(95)00518-8

Table 1. Clinical Characteristics of Patients and Normal Subjects

		Patients With NIDDM				
		Without Complications		With Complications		
	Normal Subjects (n = 46)	Good Control: Hb A_{1c} <9.0% (n = 31)	Poor Control: Hb A_{1c} ≥9.0% (n = 23)	Good Control: Hb A_{1c} <9.0% (n = 25)	Poor Control: Hb A_{1c} ≥9.0% (n = 17)	Patients With IDDM (n = 8)
Male/female	21:25	14:17	10:13	10:15	8:9	1:7
Age (yr)	58.3 ± 2.4	61.8 ± 2.1	58.2 ± 2.2	62.9 ± 2.5	64.6 ± 2.0	42.8 ± 3.6
Duration of diabetes (yr)	—	7.8 ± 1.2	9.2 ± 1.3	13.9 ± 1.4	19.6 ± 2.5	12.9 ± 3.0
Range		0.6–26	0.6–23	0.6–30	5–32	5–28
Current therapy						
Diet	—	16	5	4	0	0
Oral hypoglycemic agents	—	15	18	10	7	0
Insulin	—	0	0	11	10	8
Plasma						
FBS (mmol/liter)	5.8 ± 0.4	7.5 ± 0.6	12.1 ± 0.8*	6.2 ± 0.3	9.6 ± 0.5†	13.6 ± 2.4†
Hb A_{1c} (%)	5.8 ± 0.2	7.1 ± 0.3	11.3 ± 0.3*	7.0 ± 0.2	10.5 ± 0.4*	8.1 ± 0.5
Cholesterol (mmol/liter)	4.76 ± 0.16	4.89 ± 0.16	5.04 ± 0.26	4.63 ± 0.20	5.06 ± 0.35	5.09 ± 0.20
Triglycerides (mmol/liter)	1.17 ± 0.08	1.10 ± 0.09	1.19 ± 0.14	1.14 ± 0.10	1.44 ± 0.19	0.98 ± 0.13
HDL cholesterol (mmol/liter)	1.42 ± 0.13	1.11 ± 0.26	1.16 ± 0.13	1.29 ± 0.12	1.19 ± 0.13	1.51 ± 0.08

*$p < 0.01$, †$p < 0.05$, significantly different from normal subjects. Data presented are mean value ± SE, range or number of patients. FBS = fasting blood sugar level; Hb A_{1c} = hemoglobin A_{1c}; HDL = high density lipoprotein; IDDM = insulin-dependent diabetes mellitus; NIDDM = non–insulin-dependent diabetes mellitus.

Methods

Patients. The present study included 96 patients with non–insulin-dependent diabetes mellitus (42 men, 54 women; mean [±SD] age 61.6 ± 1.4 years, range 18 to 69) (Table 1). Diabetes was diagnosed according to the World Health Organization criteria (25). Fifty-four patients with non–insulin-dependent diabetes mellitus had no diabetic complications (e.g., diabetic retinopathy, diabetic nephropathy, diabetic neuropathy), and 42 had one or more diabetic complications. Retinopathy was classified as normal, preproliferative retinopathy or proliferative retinopathy by an ophthalmologist, and preproliferative or proliferative retinopathy was defined as diabetic retinopathy. Diabetic nephropathy was defined by the presence of persistent proteinuria (>0.5 g/day) with increased serum concentrations of creatinine (>1.8 mg/dl). Diabetic neuropathy was defined by the presence of polyneuropathy with a motor nerve conduction velocity of the fibular nerve <39.3 m/s or absence of the Achilles tendon reflex. Patients were classified as having either poor (hemoglobin [Hb] A_{1c} ≥9.0%) or good glycemic control (Hb A_{1c} <9.0%). The duration of diabetes was longer in patients with non–insulin-dependent diabetes mellitus with than without complications, but the difference was not significant.

Forty-six healthy nondiabetic subjects matched for age and gender served as the normal control group (21 men, 25 women; mean 58.3 ± 2.4 years, range 29 to 67). In addition, eight patients with insulin-dependent diabetes mellitus (one man, seven women mean age 42.8 ± 3.6 years, range 16 to 61; Hb A_{1c} ≥9.0%, two patients; Hb A_{1c} <9.0%, six patients) were also studied. Patients with underlying thyroid, renal or liver disease; hyperlipidemia; ischemic heart disease; heart failure; hypertension; inflammatory disease; and malignancies were excluded from both the diabetic and nondiabetic groups. Use of drugs that could affect platelet function, blood coagulation and lipid metabolism was prohibited during the 2 weeks before blood collection. A fasting blood sample was collected at 9 AM after the subject had rested for at least 1 h, and thrombin generation was measured as described later. Smoking was prohibited for at least 2 h before blood collection. Fasting blood sugar and serum cholesterol, triglyceride and high density lipoprotein (HDL) cholesterol levels were also measured. In addition, the plasma levels of TAT, plasmin-alpha$_2$ plasmin-inhibitor complex (PIC) and tissue-plasminogen activator t-PA antigen were also determined at the same time. All subjects gave written informed consent to participate in this study.

Measurement of platelet-dependent thrombin generation. Thrombin generation was measured according to the method of Aronson et al. (24), with slight modifications. Venous blood was collected into tubes containing sodium citrate (3.8% sodium citrate/blood 1:9) and was centrifuged at 120*g* at 22°C for 10 min. Platelet-rich plasma was separated from the upper two-thirds of the supernatant to avoid the contamination of other cells, including monocytes, after which the residual blood was centrifuged at 1,500*g* for 15 min to obtain platelet-poor plasma. Platelet counts in platelet-rich plasma were determined with a Coulter counter (S-Plas IV, Coulter Electronics), and the platelet concentration was adjusted to 150 × 10^9/liter with platelet-poor plasma after absence of contamination by other cells had been confirmed. Aliquots of 0.5 ml platelet-rich plasma were placed into round-bottomed polypropylene tubes (12 × 75 mm), and 20 μl of 1 mol/liter calcium carbonate was added to start clotting. The 10-μl of samples were added to wells of a microtiter plate containing 90 μl of 3.8% sodium

citrate at 10-min intervals up to 60 min, and the clotting time was determined for each sample. The clotted platelet-rich plasma sample changed to a spherical clot floating in the plasma on gentle vortex mixing, and the liquid sample could be readily aspirated. After completion of the sample addition, 50 μl of 0.5 mmol/liter S-2238 (H-D-Phe-Arg-NH-NO_2-2HCl, a thrombin-specific substrate; Daiichi Kagaku Yakuhin) in 1 mol/liter Tris, pH 8.1, was added, and the absorbance of the released color product at 2 min after the addition of S-2238 was measured spectrophotometrically at a wavelength of 405 nm using a Vmax microtiter plate reader (Easy Reader, EAR 340AT, SLT Labinstruments GmbH). Measurements at each time point were performed in triplicate. The amounts of thrombin generated were calculated from a standard curve for thrombin.

Effect of glucose on platelet-dependent thrombin generation in normal subjects. The effect of glucose on thrombin generation was investigated by measuring the platelet-dependent thrombin generation after incubating platelet-rich plasma obtained from normal subjects (n = 8, fasting blood sugar 4.6 to 5.7 mmol/liter) with various concentrations of glucose for 3 h. Glucose (dextrose, Ohtsuka Pharmaceutical Co. Ltd.) was dissolved in physiologic saline and added to the platelet-rich plasma of the normal subjects at final concentrations of 5.6, 11.1 and 22.4 mmol/liter. Physiologic saline was used as the control.

Effect of plasma from patients with non–insulin-dependent diabetes mellitus on platelet-dependent thrombin generation in normal subjects. Effects of platelet-poor plasma from patients with non–insulin-dependent diabetes mellitus without complications and from normal subjects on the platelet-dependent thrombin generation in normal control subjects were examined. The platelet concentration of platelet-rich plasma from each of the normal control subjects (n = 18) was adjusted to 150 × 10^9/liter, with platelet-poor plasma from each of the normal control subjects plus platelet-poor plasma from one patient with non–insulin-dependent diabetes mellitus or normal subject (Hb A_{1c} ≥9.0%, n = 6; Hb A_{1c} <9.0%, n = 6; normal subjects, n = 6). The volume ratio of platelet-poor plasma from the patients with non–insulin-dependent diabetes mellitus and normal subjects in the control platelet-rich plasma was adjusted to 30% and 70%. The platelet-rich plasma from normal control subjects without exogenous platelet-poor plasma served as the control. The platelet-dependent thrombin generation in the normal control subjects was measured in 18 different experiments.

Assay of plasma TAT, PIC and t-PA antigen. Plasma TAT and PIC levels were determined by enzyme immunoassay using commercially available kits (Enzygnost-TAT, Behringwerke AG, and PIC-test). Plasma t-PA antigen was determined by a solid-phase enzyme immunoassay using a commercially available kit (ELISA-tPA, Technoclone).

Statistical analysis. Statistical analysis was performed using repeated-measures analysis of variance for comparing thrombin generation. When a difference of $p < 0.05$ was shown by this procedure, multigroup comparison was performed at each time point using the Bonferroni method. The Student *t* test was used for the other analysis in the present study. A probability value <0.05 was considered statistically significant. These statistical calculations were performed using StatView-J (version 4.02, Abacus Concepts, Inc.). Results are expressed as mean value ± SE.

Results

Clinical variables. The fasting blood sugar and Hb A_{1c} levels were significantly higher in the poorly controlled non–insulin-dependent diabetes mellitus groups with or without complications than in the well controlled groups and the healthy group. However, no significant differences among these groups were noted with regard to the serum levels of total cholesterol, triglycerides or HDL cholesterol (Table 1).

Platelet-dependent thrombin generation in patients with non–insulin-dependent diabetes mellitus without diabetic complications. The clotting time measured in platelet-rich plasma (Fig. 1A) was significantly shorter ($p < 0.05$) in both diabetic groups without complications than in the healthy group (Table 2), although there was no significant difference between the patients with good and poor glycemic control. The data for thrombin generation in platelet-rich plasma for each diabetic group without complications are shown in Fig. 1A. Thrombin generation revealed the greatest level in the poorly controlled diabetic group, followed by the well controlled diabetic group and the healthy group, and the differences among the three groups were statistically significant ($p < 0.001$). Multigroup comparisons of thrombin generation demonstrated a significant difference between the poorly controlled diabetic group and the healthy group ($p < 0.001$) and between the well and poorly controlled diabetic groups ($p < 0.01$). Concerning the data for each measurement time, thrombin generation was significantly greater in the poorly controlled group than in the healthy group from 10 min after the addition of calcium chloride (10 min, $p < 0.05$; 20 min, $p < 0.01$; 30 min, $p < 0.001$; 40 min, $p < 0.001$; 50 min, $p < 0.001$; 60 min, $p < 0.001$). In the poorly controlled diabetic group, thrombin generation was also significantly greater than in the well controlled group from 10 min after the addition of calcium chloride (10 min, $p < 0.05$; 20 min, $p < 0.05$; 30 min, $p < 0.05$; 40 min, $p < 0.01$) (Fig. 1A). The well controlled group was divided into subgroups with an Hb A_{1c} <7.5% and 7.5% to 9%, and the clotting time and thrombin generation were compared between these subgroups. This comparison showed no significant difference between the two subgroups.

Platelet-dependent thrombin generation in patients with non–insulin-dependent diabetes mellitus with complications. Clotting time was significantly shorter ($p < 0.05$) in the poorly controlled diabetic group with complications (Fig. 1B) than in the healthy group (Table 2), although there was no significant difference between the well controlled diabetic patients with complications and the healthy group. Thrombin generation achieved the greatest level in the poorly controlled diabetic group with complications, followed by the well controlled

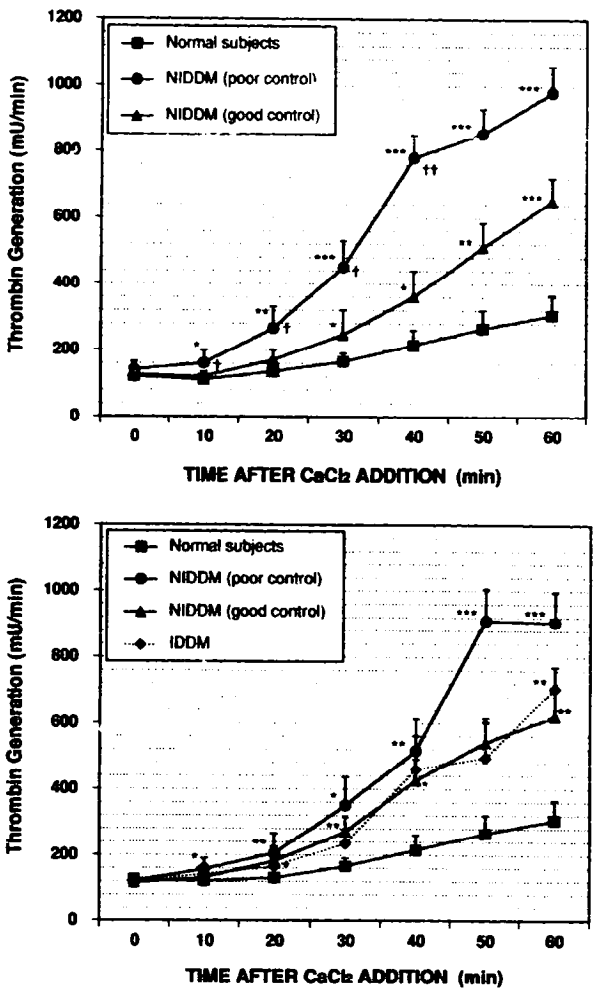

Figure 1. Platelet-dependent thrombin generation in patients with non–insulin-dependent (NIDDM) and insulin-dependent diabetes mellitus (IDDM). **A,** Thrombin generation revealed high levels in patients with poorly controlled non–insulin-dependent diabetes mellitus without complications compared with those in normal subjects, but its value was closer to that in normal control subjects and in patients with well controlled non–insulin-dependent diabetes mellitus without complications. **B,** Thrombin generation was also greatest in patients with poorly controlled non–insulin-dependent diabetes mellitus with complications and intermediate in patients with well controlled non–insulin-dependent diabetes mellitus with complications, as in the case of patients with non–insulin-dependent diabetes mellitus without complications. Thrombin generation in patients with insulin-dependent diabetes mellitus revealed high levels compared with those in normal subjects but was not as high as those in patients with poorly controlled non–insulin-dependent diabetes mellitus. Results are shown as mean value ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, normal subjects versus each diabetic group. †$p < 0.05$, ††$p < 0.01$, diabetic groups with good versus poor control.

diabetic group and the healthy group, and the differences among the three groups were statistically significant ($p < 0.001$) (Fig. 1B). Multigroup comparisons of thrombin generation demonstrated a significant difference between the poorly controlled diabetic group with complications and the healthy group ($p < 0.001$), as in the case of the poorly controlled diabetic group without complications and the healthy group. However, no significant difference was observed between the poorly and well controlled diabetic groups with complications. There were no significant differences in thrombin generation between the poorly controlled diabetic group with complications and that without complications or between well controlled diabetic patients with and without complications. Concerning the data for each measurement time, thrombin generation was significantly greater in the poorly controlled group than in the healthy group from 10 min after the addition of calcium chloride (10 min, $p < 0.05$; 20 min, $p < 0.01$; 30 min, $p < 0.05$; 40 min, $p < 0.01$; 50 min, $p < 0.001$, 60 min, $p < 0.001$). In the well controlled diabetic group, thrombin generation was significantly greater than that in the healthy group (30 min, $p < 0.01$; 40 min, $p < 0.05$; 60 min, $p < 0.01$) (Fig. 1B).

Platelet-dependent thrombin generation in patients with insulin-dependent diabetes mellitus. Clotting time and thrombin generation in the eight patients with insulin-dependent diabetes mellitus were shorter and higher than those in the healthy control subjects, respectively. However, thrombin generation was not as great as that in the poorly controlled diabetic group (Fig. 1B). Thrombin generation was significantly different between the patients with insulin-dependent diabetes mellitus and normal subjects at each time point (20 min, $p < 0.05$; 60 min, $p < 0.01$) (Fig. 1B).

Effect of glucose on platelet-dependent thrombin generation in normal subjects. When platelet-rich plasma samples from normal subjects were preincubated with glucose at concentrations of 5.6 to 22.4 mmol/liter for 3 h, clotting time tended to be shorter and thrombin generation became higher, according to the glucose concentration added, than that in control subjects without exogenous glucose (Table 3).

Effect of plasma from diabetic patients on platelet-dependent thrombin generation in normal subjects. Clotting time of platelet-rich plasma from normal subjects was shortened with increasing concentrations of platelet-poor plasma from patients with non–insulin-dependent diabetes mellitus without complications in both of the two glycemic control groups but was not affected by platelet-poor plasma from normal subjects (Fig. 2). The differences were significant from the control in both the well and poorly controlled diabetic groups (70% vol) (Fig. 2B,C). Platelet-dependent thrombin generation was not affected by the addition of platelet-poor plasma from normal subjects (Fig. 2A). Thrombin generation tended to become higher with increasing doses of platelet-poor plasma from patients with non–insulin-dependent diabetes mellitus compared with control subjects, especially in the poorly controlled group, and the differences were significant in both the well and poorly controlled diabetic groups (70% vol) (Fig. 2B,C).

Plasma TAT, PIC and t-PA antigen. Markers of the coagulation and fibrinolysis system were also compared among the diabetic groups (Table 2). No significant difference in plasma TAT levels was noted among these groups. The intergroup

Table 2. Coagulation and Fibrinolysis Data for Patients and Normal Subjects

		Patients With NIDDM				
		Without Complications		With Complications		
	Normal Subjects (n = 46)	Good Control: Hb A_{1c} <9.0%	Poor Control: Hb A_{1c} ≥9.0%	Good Control: Hb A_{1c} <9.0%	Poor Control: Hb A_{1c} ≥9.0%	Patients With IDDM (n = 8)
Clotting time (min)	48 ± 4	34 ± 4*	28 ± 4*	38 ± 7	34 ± 5*	40 ± 5
Plasma						
TAT (ng/ml)	2.8 ± 0.7	3.4 ± 0.8	2.9 ± 0.7	2.6 ± 0.7	2.8 ± 0.5	2.5 ± 0.2
PIC (μg/ml)	0.77 ± 0.09	0.81 ± 0.10	0.78 ± 0.09	0.70 ± 0.11	0.75 ± 0.10	0.80 ± 0.05
t-PA antigen (ng/ml)	2.7 ± 0.4	3.0 ± 0.5	2.1 ± 0.4	2.7 ± 0.5	2.5 ± 0.4	2.9 ± 0.1

*$p < 0.05$, significantly different from normal subjects. Data presented are mean value ± SE. PIC = plasmin-alpha$_2$ plasmin inhibitor; TAT = thrombin-antithrombin-III complex; t-PA = tissue-type plasminogen activator; other abbreviations as in Table 1.

differences were also not significant for plasma PIC levels. The t-PA antigen levels were slightly lower in the poorly controlled group without complications than in the healthy group, but again there were no significant intergroup differences.

Discussion

It is generally agreed that coagulability is more or less enhanced in diabetes mellitus (10–18). In the present study, platelet-dependent thrombin generation was measured in diabetic patients with good and poor glycemic control. Significantly increased thrombin generation was found in both diabetic groups compared with that in healthy subjects.

Significance of measurement of platelet-dependent thrombin generation. A decreased plasma level of antithrombin-III (10,11,13), which is reported to result from nonenzymatic glycosylation, and an increased plasma level of TAT (16–18) have been reported as indicators of hypercoagulability in diabetes. However, opposing data have also been published (3,9–22), and there is no consensus as yet. This lack of consensus may be partly because coagulation markers are assayed in ordinary plasma rather than in platelet-rich plasma. Thrombus formation proceeds as a result of interactions between platelets and the coagulation system. Briefly, coagulation factor X generation occurs on the activated platelet surfaces, and platelet factor III accelerates the conversion of prothrombin to thrombin (26,27). Activation of coagulation factor XI on the platelet surface is considered to trigger the coagulation cascade (28). The small amounts of thrombin generated during the coagulation cascade serve to activate platelets and stimulate the release of coagulation factor Va from the alpha-granules. Coagulation factor Va forms a complex with activated coagulation factor X, and thrombin is thus generated very efficiently and quickly on the activated platelet surface. Thus, platelets and thrombin act complementarily in the process of thrombus formation, and the coagulation cascade is thought to be remarkably augmented on the platelet membrane (23). Therefore, measurement of thrombin generation in platelet-rich states, as was performed in the present study, is thought to provide more accurate data concerning the coagulation system. Because thrombin binds not only to antithrombin-III but also to heparin cofactor II and thrombomodulin, TAT is a less than perfect indicator of thrombin generation. Thus, coagulation markers such as antithrombin-III and TAT are only indirect indicators of thrombin generation. Plasma TAT levels in the diabetic patients assessed in the present study did, in fact, vary widely, and there were no significant intergroup differences. Measurement of platelet surface thrombin activity may therefore be more useful for the investigation of coagulation. The results of the present study clearly confirmed the existence of hypercoagulability in diabetic patients.

Clinical implications. In the present study, the clotting time and platelet-dependent thrombin generation were assessed separately in groups with good and poor glycemic control on the basis of Hb A_{1c} levels. The results in the well controlled diabetic group were intermediate, regardless of whether complications were present or not, between that of the poorly controlled group and the healthy subjects, suggesting normalization of clotting time and thrombin activity by improving glycemic control. A significant difference in thrombin generation was observed between the poorly and well controlled diabetic groups without complications, but there was no significant difference between the poorly and well controlled diabetic patients with complications. It is possible that diabetic patients with complications might possess some unknown factors that can affect thrombin generation, and this could result in no significant difference in thrombin generation between poor and good glycemic control. Diabetic patients with complications may thus not be appropriate study patients

Table 3. Effects of Glucose on Clotting Time and Platelet-Dependent Thrombin Generation in Normal Subjects*

Glucose Added (mmol/liter)	Clotting Time (min)	Thrombin Generation 30 min After Calcium Chloride Added (mU/min)
0	46 ± 7	98 ± 16
5.6	45 ± 4	122 ± 22
11.1	38 ± 4	178 ± 35†
22.4	36 ± 5†	185 ± 24†

*Platelet-rich plasma in normal subjects was preincubated with glucose for 3 h. †$p < 0.05$, significantly different from control without exogenous glucose. Data presented are mean value ± SE.

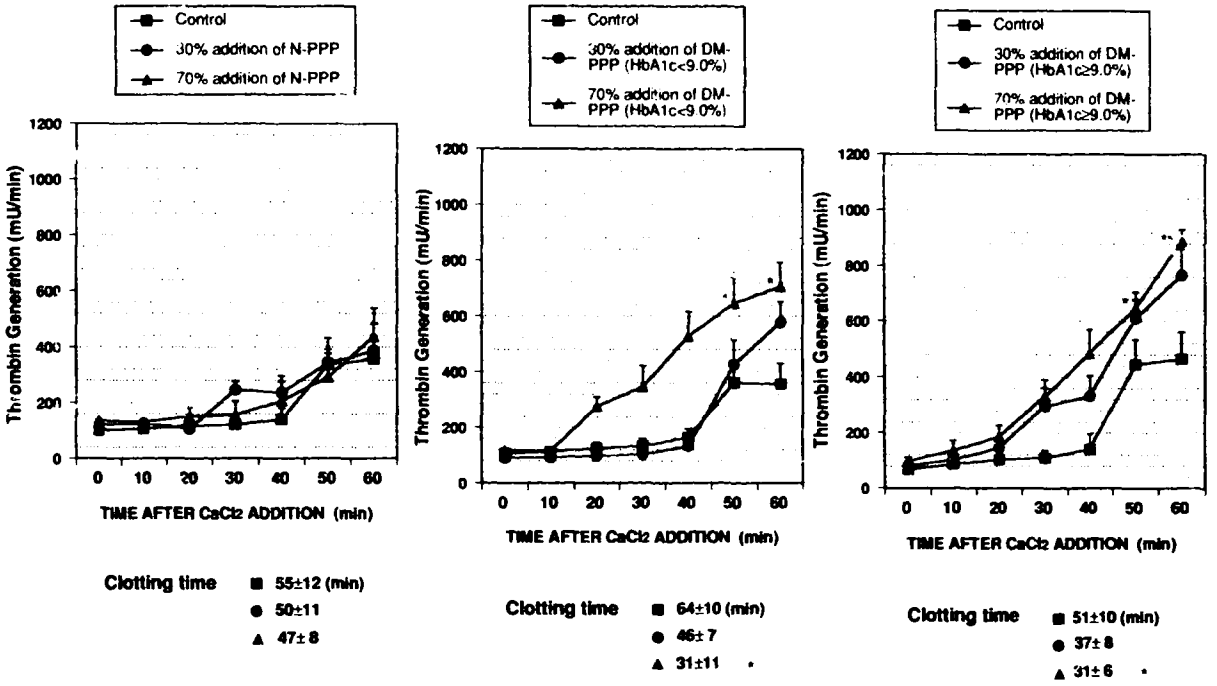

Figure 2. Effect of platelet-poor plasma (PPP) from patients with non-insulin-dependent diabetes mellitus (NIDDM) on platelet-dependent thrombin generation in normal subjects. **A**, Clotting time and platelet-dependent thrombin generation were unaffected by the addition of platelet-poor plasma obtained from normal subjects (N-PPP) (n = 6). **B** and **C**, Clotting time and thrombin generation were shorter and higher, respectively, in accordance with increasing concentrations of platelet-poor plasma obtained from patients with non-insulin-dependent diabetes mellitus (DM-PPP) (**B**, Hb A_{1c} <9.0%, n = 6; **C**, Hb A_{1c} ≥9.0%, n = 6). Results shown are mean value ± SE. *p < 0.05, **p < 0.01, significantly different from control.

for investigating thrombin generation in diabetes mellitus. There are few studies that have classified diabetic patients by their level of glycemic control and assessed various coagulation markers (12,17). Small et al. (12) reported that fibrinopeptide A levels were higher in diabetic patients than in nondiabetic subjects and that there was an inverse correlation between fibrinopeptide A and Hb A_{1c} (12). They suggested that thrombin activity was reduced by better control of diabetes. According to Van Wersch et al. (17), the TAT level was abnormally high in diabetic patients and was higher in the poorly controlled group than in the well controlled group Hb A_{1c} >90% and <10%, respectively). The present study yielded data consistent with these reports for platelet-dependent thrombin activity. These findings suggested that good glycemic control may lead to the inhibition of a hypercoagulabie state in diabetic patients.

Limitations of the study. There are several limitations to the present study.

1. We did not investigate the effects of obesity on thrombin generation, although we excluded subjects with other underlying diseases in both groups of diabetic patients. Many recent studies (29–33) have suggested involvement of the coagulation system in hyperlipidemia, one of the risk factors for atherosclerosis, and considerable data are available showing enhanced platelet function and hypercoagulability in patients with hyperlipidemia. We also investigated platelet-dependent thrombin generation in patients with hyperlipidemia and found that thrombin generation was increased, as it is in diabetic patients (unpublished observations). However, only diabetic patients without hyperlipidemia were enrolled in the present study, so the possibility of an effect of hyperlipidemia on thrombin generation can be ruled out.
2. Patients with non–insulin-dependent diabetes mellitus in the present study were obtained randomly and classified into two groups by Hb A_{1c} levels. Platelet-dependent thrombin generation should be compared in the same diabetic patients before and after achievement of glycemic control. Such investigations may provide more accurate assessment in coagulability in diabetic patients.
3. We failed to find any significant difference between the well and poorly controlled diabetic patients regarding platelet-dependent thrombin generation at ≥50 min after calcium chloride addition, showing the total extent of prothrombin activation to be unchanged. This may reflect the extent of coagulation factor Xa/Va activity.
4. We failed to detect higher levels of thrombin generation in patients with insulin-dependent diabetes mellitus compared with those in patients with poorly controlled non–insulin-dependent diabetes mellitus. This finding probably reflects the good glycemic control in insulin-dependent diabetes mellitus in the present study.

5. We were unable to evaluate the mechanism of hypercoagulability in non–insulin-dependent diabetes mellitus. In the present study, thrombin generation in platelet-rich plasma from normal subjects tended to be increased with increasing doses of platelet-poor plasma from patients with non–insulin-dependent diabetes mellitus in both the well and poorly controlled groups. These findings suggest that hypercoagulability in non–insulin-dependent diabetes mellitus could result from certain plasma factors in non–insulin-dependent diabetes mellitus. We were unable to identify stimulatory or inhibitory effects of metabolites, such as insulin, uric acid or kynurenic acid, the levels of which are variable in diabetic patients, on the platelet-dependent thrombin generation in normal subjects (data are not shown). However, thrombin generation in platelet-rich plasma from normal subjects was stimulated by the addition of glucose in vitro, so that the higher levels of thrombin generation occurring in patients with non–insulin-dependent diabetes mellitus are presumably related in part to some direct action of glucose. Moreover, it is possible that diabetic patients possess some type of factor that can facilitate coagulability, although the details remain unclear at present.

Conclusions. Determination of platelet-dependent thrombin generation suggested that coagulability was enhanced in diabetic patients compared with that in healthy subjects. Enhanced thrombin generation may be one reason for the progression of atherosclerosis. The present study also showed that thrombin generation can be reduced by good control of blood glucose. Therefore, our results suggest that good glycemic control is important for prevention of atherosclerosis and thrombosis and may also improve the overall prognosis of patients with diabetes.

References

1. Ostermann H, van de Loo J. Factors of the hemostatic system in diabetic patients. A survey of controlled studies. Haemostasis 1986;16:386–416.
2. Colwell JA, Halushka PV. Platelet function in diabetes mellitus. Br J Haematol 1980;44:521–6.
3. Di Mario U, Borsey DQ, Contreas G, Prowse CV, Clarke BF, Andreani D. The relationship of soluble immune complexes, insulin antibodies and insulin-anti-insulin complexes to platelet and coagulation factors in type I diabetic patients with and without proliferative retinopathy. Clin Exp Immunol 1986;65:57–65.
4. Frade LJG, de la Calle H, Alava I, Navarro JL, Creighton LJ, Gaffney PJ. Diabetes mellitus as a hypercoagulable state: its relationship with fibrin fragments and vascular damage. Thromb Res 1987;47:533–40.
5. Udvardy M, Torok I, Rak K. Plasma thoromboxane and prostacyclin metabolite ratio in atherosclerosis and diabetes mellitus. Thromb Res 1987;47:479–84.
6. Ramani A, Kumar S, Tyagi AN. Effect of serum cholesterol on platelet adhesiveness in vascular complications of diabetes mellitus. Indian Heart J 1988;40:144–7.
7. Cho NH, Becker D, Dorman JS, et al. Spontaneous whole blood platelet aggregation in insulin-dependent diabetes mellitus: an evaluation in an epidemiologic study. Thromb Haemostas 1989;61:127–30.
8. Davi G, Catalano I, Averna M, et al. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. N Engl J Med 1990;322:1769–74.
9. Toth L, Szenasi P, Jambor G, Kammerer L, Romics L. Platelet function in male diabetics with and without macrovascular complications. Diabetes Res Clin Pract 1992;15:143–8.
10. Ceriello A, Russo PD, Zuccotti C, Florio A, Pietrantuono C, Rosato GB. Decreased antithrombin III activity in diabetes may be due to non-enzymatic glycosylation—a preliminary report. Thromb Haemostas 1983;50:633–4.
11. Brownlee M, Vlassara H, Cerami A. Inhibition of heparin-catalyzed human antithrombin III activity by nonenzymatic glycosylation. Diabetes 1984;33:532–5.
12. Small M, Lowe GDO, MacCuish AC, Forbes CD. Thrombin and plasmin activity in diabetes mellitus and their association with glycaemic control. Q J Med 1987;65:1025–31.
13. Villanueva GB, Allen N. Demonstration of altered antithrombin III activity due to nonenzymatic glycosylation at glucose concentration expected to be encountered in severely diabetic patients. Diabetes 1988;37:1103–7.
14. Marongiu F, Conti M, Mameli G, et al. Is the imbalance between thrombin and plasmin activity in diabetes related to the behaviour of antiplasmin activity? Thromb Res 1990;58:91–9.
15. Ceriello A, Giugliano D, Quatraro A, Marchi E, Barbanti M, Lefebvre P. Evidence for a hyperglycaemia-dependent decrease of antithrombin III–thrombin complex formation in humans. Diabetologia 1990;33:163–7.
16. Rose D, Kotschy M, Rewakoviez M, Listopadzki D. Thrombin/antithrombin III complex in patients with peripheral occlusive arterial disease. Fol Haematol 1990;117:405–11.
17. Van Wersch JWJ, Westerhuis LWJJM, Venekamp WJRR. Coagulation activation in diabetes mellitus. Haemostasis 1990;20:263–9.
18. Carmassi F, Morale M, Puccetti R, et al. Coagulation and fibrinolytic system impairment in insulin dependent diabetes mellitus. Thromb Res 1992;67:643–54.
19. Knaap JH, de Boer AC, Pannebakker MAG, van Heerde W, den Ottolander GJH. The effect of diabetes regulation on platelet release, fibrinolysis and coagulation tests, before and after stimulation with DDAVP. Thromb Haemostas 1985;53:118–21.
20. Dallinger KJC, Jennings PE, Toop MJ, Gyde OHB, Barnett AH. Platelet aggregation and coagulation factors in insulin dependent diabetics with and without microangiopathy. Diabet Med 1987;4:44–8.
21. Christe M, Fritschi J, Lammle B, et al. Fifteen coagulation and fibrinolysis parameters in diabetes mellitus and in patients with vasculopathy. Thromb Haemostas 1984;52:138–43.
22. Patrassi GM, Picchinenna R, Vettor R, Cappellato G, Coccarielli D, Girolami A. Antithrombin III activity and concentration in diabetes mellitus. Thromb Haemostas 1985;54:415–7.
23. Zwaal RFA, Comfurius P, Bevers EM. Platelet procoagulant activity and microvesicle formation: its putative role in hemostasis and thrombosis. Biochim Biophys Acta 1992;1180:1–8.
24. Aronson DL, Chang P, Kessler CM. Platelet-dependent thrombin generation after in vitro fibrinolytic treatment. Circulation 1992;85:1706–12.
25. World Health Organization Expert Committee. Second Report on Diabetes Mellitus. Geneva, World Health Organization 1980 (Tech. Rep. Ser., no. 646).
26. Cox AC, Inyangetor P, Esmon CT, White BN. The effect of aggregation and release on platelet prothrombin-converting activity. Blood 1979;54:659–72.
27. Rosing J, van Rijn JLML, Bevers EM, van Dieijen G, Comfurius P, Zwaal RFA. The role of activated human platelets in prothrombin and factor X activation. Blood 985;65:319–32.
28. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 1990;76:1–16.
29. Shattil SJ, Anaya-Galindo R, Bennett J, Colman RW, Cooper RA. Platelet hypersensitivity induced by cholesterol incorporation. J Clin Invest 1975;55:636–43.
30. Davi G, Averna M, Novo S, et al. Effects of synvinolin on platelet aggregation and thromboxane B_2 synthesis in type IIa hypercholesterolemic patients. Atherosclerosis 1989;79:79–83.
31. Mazeaud MM, Driss F, Sang K-HLQ, et al. Biochemical and functional alterations associated with hypercholesterolemia in platelets from hypertensive patients. Atherosclerosis 1992;94:201–11.
32. Wada H, Mori Y, Kaneko T, et al. Hypercoagulable state in patients with hypercholesterolemia: effects of pravastatin. Clin Ther 1992;14:829–34.
33. Bo M, Bonino F, Neirotti M, et al. Hemorheologic and coagulative pattern in hypercholesterolemic subjects treated with lipid-lowering drugs. Angiology 1991;42:106–13.